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Valorizing food wastes: assessment of novel yeast strains for enhanced production of single-cell protein from wasted date molasses

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Abstract

In the current study, several non-conventional yeast strains were screened and adapted to produce single-cell protein (SCP) at high productivities and yields from wasted date molasses (WDM). Among the tested yeasts, *Hanseniaspora guilliermondii* JQ690237, *Hanseniaspora uvarum* JQ690236, *Issatchenkia orientalis* JQ690240, and *Cyberlindnera fabianii* JQ690242 emerged as the highest producers of biomass during small-scale batch experiments, leading to yields up to 700 g dry biomass/kg of WDM after 48 h of incubation. It was shown that the supplementation of the WDM medium with either organic or inorganic nitrogen sources, enhanced significantly the bioconversion efficiency of WDM into single-cell protein, with *H. guilliermondii* and *I. orientalis* exhibiting the highest production of biomass, with a protein content of up to 54.3%. The scaling up of the process confirmed its efficiency, indicating that newly isolated yeasts are promising SCP producers for possible industrial exploitation of the specific waste toward animal feed.

Keywords Wasted date molasses · Single-cell protein · Yeast · 26S rDNA · *Hanseniaspora* · *Issatchenkia* · *Cyberlindnera*

1 Introduction

In developing countries, the constantly increasing rates of population growth and food production needs, have led to a widening gap between supply and demand [1, 2]. Inevitably such a gap results in the malnutrition of large population groups and is associated with protein deficiency diseases, making thus imperative the need for identifying new ways for the production of alternative protein sources, such as microorganisms [3, 4]. Single-cell protein (SCP) production technology may contribute to altering the worldwide protein shortage problem, via focusing on the exploitation of microbes as a potential protein source for both human nutrition and as animal feed [5, 6]. Into this context, microorganisms serve as effective biocatalysts able to bioconvert low-value substrates, such as food wastes, into a valuable final product with added nutritional and market value [7, 8].

Although SCP can be produced by different types of microorganisms, fungi and bacteria are generally preferred [9, 10]. Indeed, their rapid growth rates and high protein content, as well as their ability to grow efficiently on low-cost substrates, have made them the prime candidates as SCP producers [4]. Yeasts are fungal-derived SCP commonly included in animal and aquaculture feeds with excellent results, and a 10% contribution of this product is normally accepted in mixtures with other sources providing carbohydrates, lipids, and vitamins [11, 12]. The quality of yeast protein is considered excellent, and almost equivalent to the quality of soybean protein, being both rich in lysine [4]. Similarly to some plant proteins, yeast protein is low in sulfur amino acids, but supplementing dried yeast with 0.5% methionine can raise its protein quality up to that of casein [13]. Yeasts that have been approved for SCP production include the species *Candida utilis*, *Candida robusta*, *Candida tropicalis*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Saccharomyces*

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cerevisiae, and *Yarrowia lipolytica* [4, 12, 14–16]. Besides the use of those yeasts, it can be assumed though that via a wide screening of new yeasts more efficient microorganisms in the processing of SCP production can be identified and exploited, aiming to enhance SCP productivity and explore different classes of proteins. In the current study, the yeasts *Hanseniaspora guilliermondii*, *Hanseniaspora uvarum*, *Issatchenkia orientalis*, and *Cyberlindnera fabianii* were evaluated as SCP hyper-producers after their isolation and comparative evaluation with more than 140 new isolates. Although their proposed utilization for protein production is introduced for the first time and all four species could be considered food-safe, since as the existing literature suggests, they are food-related species and/or have applications in the production of edible fermentation products. More specifically, *H. uvarum* and *H. guilliermondii* are principal yeast species found in spontaneous fermentation during wine making and can influence the chemical composition of the wine resulting in better and more complex aroma [17]. *I. orientalis* has been detected in a variety of beverage fermentations, such as the tumultuous or late stage of natural fermentation of wine fermentation and in the tequila production process, whereas its presence in traditional sourdough and milk fermentation products producing flavor compounds has been reported [18]. The literature on *C. fabianii* indicating its food-related safety is scarcer, reporting its occurrence on traditional fermented beverages [19, 20]. However, it should be pointed out that the suitability of any type of SPC as food or food supplement must be considered individually and always in accordance with national and international standards for food safety.

The substrate used and the selection of biocatalyst represent, indeed, the fundamentals of the SCP production process. Into this context, the quest for new low-cost and highly bioconvertable substrates as well as effective and easy to handle microorganisms remains constant, aiming to obtain high productivities of the SCP in a cost-efficient manner [21, 22]. The main factors that affect the SCP production by microorganisms and are crucial for the sustainability of the overall process are incubation time, temperature, pH, and aeration as well as the carbon and nitrogen sources [23]. The latter can be obtained from different wastes and residual biomass and there is a wide range of both that may fit the necessary criteria that may ensure sustainable SCP production, i.e., being abundant, renewable, nontoxic, inexpensive, and able to support rapid growth of the selected biocatalysts [24, 25].

Fruits are dietary products of high nutritional value, but since they have a short shelf life and are vulnerable to spoilage if not consumed timely, they end up discernable, thus contributing significantly to food losses and food waste accumulation [26, 27]. On the other hand, unlike other types of food wastes that require pretreatment and/or hydrolysis to be bioconverted [28–30], fruit wastes due to the high content of easily fermentable sugars that can be a substrate for a variety of microorganisms can serve as excellent substrates for microbial growth. In order to preserve the sugar content in such vulnerable to degradation substrates until bioconversion, different ways may be applied including drying and concentration through which water activity decreases to levels that allow their long-term maintenance [31].

Into this context, in the current study fresh dates that were spoiled and would be discarded, were collected and processed via thermal concentration to obtain their sugar content in the form a thick solution, i.e., date molasses (DM). DM is a term that is applied generally to denote a dark sticky date syrup (dibs) that is the common derived by-product during dates' manufacturing [32], containing high amount of reducing sugars, low protein content, and several minerals [33]. In the current study, DM was recovered from wasted (spoiled) dates and as such the final product which was called wasted DM (WDM) was used as feedstock in the current study. Based on its physicochemical characteristics DM and consequently WDM could represent an excellent medium for yeast growth and production of high quantities of biomass and single-cell protein.

The main scope of the current study was to achieve the maximum exploitation of the sugar fraction of spoiled dates toward SCP by exploring the potential of novel yeast strains and identifying the SCP production conditions. The long-term conservation of date sugars serving as carbon source was achieved by processing the discarded dates to molasses, i.e., to WDM, whereas the newly isolated strains *H. guilliermondii*, *H. uvarum*, *I. orientalis*, and *C. fabianii* were assessed as hyper-producers of SCP. To the best of our knowledge, the proposed methodology has been applied for the first time for SCP production.

2 Materials and methods

2.1 Preparation and physicochemical characteristics of the WDM

The WDM was prepared using common spoiled dates from the street market of Abha city, Kingdom of Saudi Arabia. To obtain the WDM, the method described by Doma et al. [34] was followed with minor modifications. Briefly, the flesh of wasted dates was mixed with hot water (80–90 °C) and was left for 1 h; then, the suspension was homogenized using a domestic mixer and the obtained slurry was filtered through cheese cloth via hand press. The residue pulp was re-mixed with hot water (80–90 °C) at a water/pulp ratio 1.5:1 (w/w) and re-extracted again twice through cheese cloth via hand press. Finally, the collected date juice was filtered through cheese cloth and the obtained extract was concentrated at

65 °C under a vacuum (500–600 mmHg) using a rotary evaporator to 70° brix.

2.2 Isolation of yeast and secreting for their single-cell protein production

In this study, 146 yeast strains belonging to the microbiome of different fruits and vegetables (Table S1) were isolated from homogenized biomass under aseptic conditions. The isolation was performed via the dilution plate method [35] on yeast-malt extract agar (yeast extract, 3 g/L; malt extract, 3 g/L; peptone, 5 g/L; glucose, 10 g/L; agar; 20 g/L). The isolates were purified on the same medium by streaking them 3 times and stored on slants at 4 °C for further use. Screening of the yeast isolates for their SCP production efficiency was tested in a flask experiment at 25 °C and 150 rpm for 72 h. Erlenmeyer flasks (250 mL) with 100 mL WDM solution (20%, v:v) were inoculated with 5 mL preculture (10^8 cell/mL in triplicate). After 72 h of incubation, 10 mL of the culture medium was transferred into a clean, weighed, and dry glass tube to estimate the microbial biomass.

2.3 Identification of the highest SCP-producer yeasts

Based on the biomass yield, the highest performing yeasts were selected for identification and obtaining their phylogenic position by sequencing the variable D1/D2 domain of the large subunit 26S ribosomal DNA. The total yeast genomic DNA was extracted following the protocol of Hesham and Mohamed [36]. The primers NL1 (5′–GCATAT CAATAAGCGGA GGAAAAG-3′) and NL4 (5′ GGTCCG TG TTTCAAG ACGG–3′) were used to amplify the DNA as described by Kurtzman and Robnett [35]. PCR reaction was carried out using 50 µL of GoTaq green master mix (Promega, Madison, WI, USA), 1 µL of each primer (0.5 mM), and 1 µL of the DNA template. The obtained DNA was purified using the GFXTM–PCR DNA and gel band purification kit (Amersham Biosciences), and the purified PCR was sequenced at Macrogen (Seoul, Korea). The DNA sequence was analyzed using the DNA Blast at the NCBI (http://www . ncbi.nlm.nih.gov/BLAST/), and the obtained nucleotide sequences were deposited in the GenBank under specific accession numbers.

2.4 Detection of favorable conditions for SCP production by yeasts

In order to achieve maximum SCP production by the four selected yeasts; *Hanseniaspora guilliermondii*, *Hanseniaspora uvarum*, *Issatchenkia orientalis*, and *Cyberlindnera fabianii*, their growth conditions were studied and optimized in terms of incubation time (12, 24, 36, 48, 60, 72, 84, and 96 h), temperature (20, 25, 30, 35, and 40 °C), pH (4.0, 5.0, 6.0, and 7.0), substrate concentration (10, 20, 30, 40 and 50% of WDM), nitrogen source (peptone and NH_4CL), and nitrogen concentration (0, 1, 2, 3, 4, and 5 g/L). The experiment sets were performed in shaking flasks, in triplicate ($n=3$), and in all case biomass yields (g dry biomass/100 g WDM) and protein content (%) were estimated.

2.5 Scale up experiments

To validate the SCP production process from WDM by the four selected yeasts, scale up experiments were conducted in the bioreactor BioFlo/CelliGen 115 by New Brunswick, USA, with all the necessary controls. The reactor was of 7-L capacity and the working volume was 3 L. The bioreactor was cleaned and steam sterilized at 121 °C for 15 min. In all experimented 20% of WDM supplemented with peptone, 4 g/L was used and was inoculated with 5% (v:v) of 24-h yeast preculture (10^8 cell/mL) obtained from 48-h-old culture at 25 °C grown on YPD medium. The temperature of fermentation was maintained at 30 ± 1 °C. The pH of the fermentation broth was regulated at 5.0 ± 0.1, using H_2SO_4 6 N or NaOH 6 N. The agitator speed was maintained constant throughout the experiment at 150 rpm and the airflow rate at 0.25 vvm. Samples were collected after 48 h to estimate the dry biomass and protein content of the cells.

2.6 Analytical methods

The physicochemical characteristics of the WDM were determined according to the standard method of AOAC [37]. Yeast biomass was determined in terms of cell dry mass (CDM) via oven drying at 80 °C for 24 h according to Gao et al. [38]. Protein content (%) was estimated based on the protocol of Zhang et al. [39] and the crude protein content in the cell was determined by using the method of Kjeldahl with ninhydrin detection with bovine serum albumin as standard [40]. Biomass yield was calculated as g CDM/100 g WDM.

2.7 Statistical analysis

All statistical analyses were performed using the SPSS 22.0 software (SPSS, 2013). The data were initially examined for their normality of distribution and homogeneity of variance. The significance of variation was assessed using one-way analysis of variance (ANOVA). The least significant difference (LSD) test was used at $P<0.05$ to identify the significant differences between the means among the treatments and the correlation between the biomass and protein content was estimated.

3 Results and discussion

3.1 Physicochemical characteristics of the substrate

Analysis of the WDM (Table 1) showed that it is a weak acidic medium (pH = 4.77) with 14.67% of moisture. The total dissolved solid was 77.0% containing a high amount of sugars (75.0%) and low content of protein (1.17%); however, fat or fibers were not detected. Reducing sugars comprised 73.12% as a main constituent in the WDM. These characteristics were close to those obtained by other researcher; however, variation in chemical composition especially sugar could be due to the type of date from which the WDM was made [32, 41, 42]. It is clear that availability of large quantity of reducing sugars in a substrate makes it appropriate as growing medium for yeasts, enhancing the fermentation yields toward valuable products such as SCP [43]. It could be used as a substrate replacing carbon and mineral sources in SCP production. It also contains a substantial level of nutrients that are required for the growth of microorganisms [44]. Because the majority of sugars in WDM is reducing sugars that are easily utilized by all yeasts, this makes it better than most of agro-wastes that need various pretreatments for liberating their sugars [22, 28–30]. Thus, WDM is considered an economic substrate for SCP production.

Table 1 Physicochemical characteristic of the WDM

Content	
Moisture (%)	14.67 ± 0.62
TDS (%)	77.00 ± 0.41
pH	4.77 ± 0.18
Ash (%)	1.60 ± 0.08
Fat	ND
Fiber	ND
Pectin (%)	0.15 ± 0.02
Tannin (%)	0.25 ± 0.02
Total sugar (%)	75.50 ± 0.20
Reducing sugars (%)	73.12 ± 0.32
Protein (%)	1.17 ± 0.06

ND, non-detected

3.2 Screening for SCP production and identification of the yeasts

A total of 146 yeast isolates were screened for their potential productivity of single-cell protein when grown on the WDM as a fermentation medium. The production varied widely among the yeasts (Fig. 1). Based on the obtained results, the yeasts were grouped into different categories on biomass yield basis. Only 4 isolates were characterized by biomass yield exceeding 60 g CDM/100 g WDM, which were chosen to be further studied in terms of their SCP capacity. The obtained sequences of D1/D2 region of the 26S rRNA gene of the identified yeasts were compared with the sequences of 26S rDNA regions in GenBank for each by means of BLAST search of the National Center for Biotechnology Information (NCBI) database. Alignment results revealed a similarity of up to 99–100% between the isolates KKUY-0078, KKUY-0036, KKUY-0060, and KKUY-0120 and *Hanseniaspora guilliermondii*, *Hanseniaspora uvarum*, *Issatchenkia orientalis*, and *Cyberlindnera fabianii*, respectively (Fig. 2). The nucleotide sequences of the new yeasts have been deposited in the GenBank database under specific accession numbers: JQ690237, JQ690236, JQ690240, and JQ690242, respectively. The sequencing of the D1/D2 of the large subunit 26S ribosomal DNA is a widely accepted technique as a standard procedure for yeast identification because a 600-bp length of the D1/D2 domain of the 26S rRNA gene contains

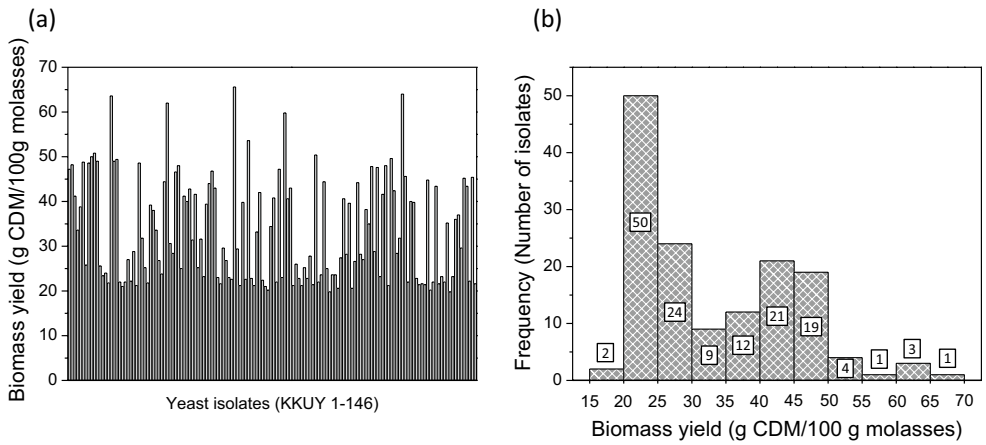

Fig. 1 Biomass yields (g CDM/100 g molasses) of the 146 isolates (**a**) and number of isolates per biomass yield range (**b**)

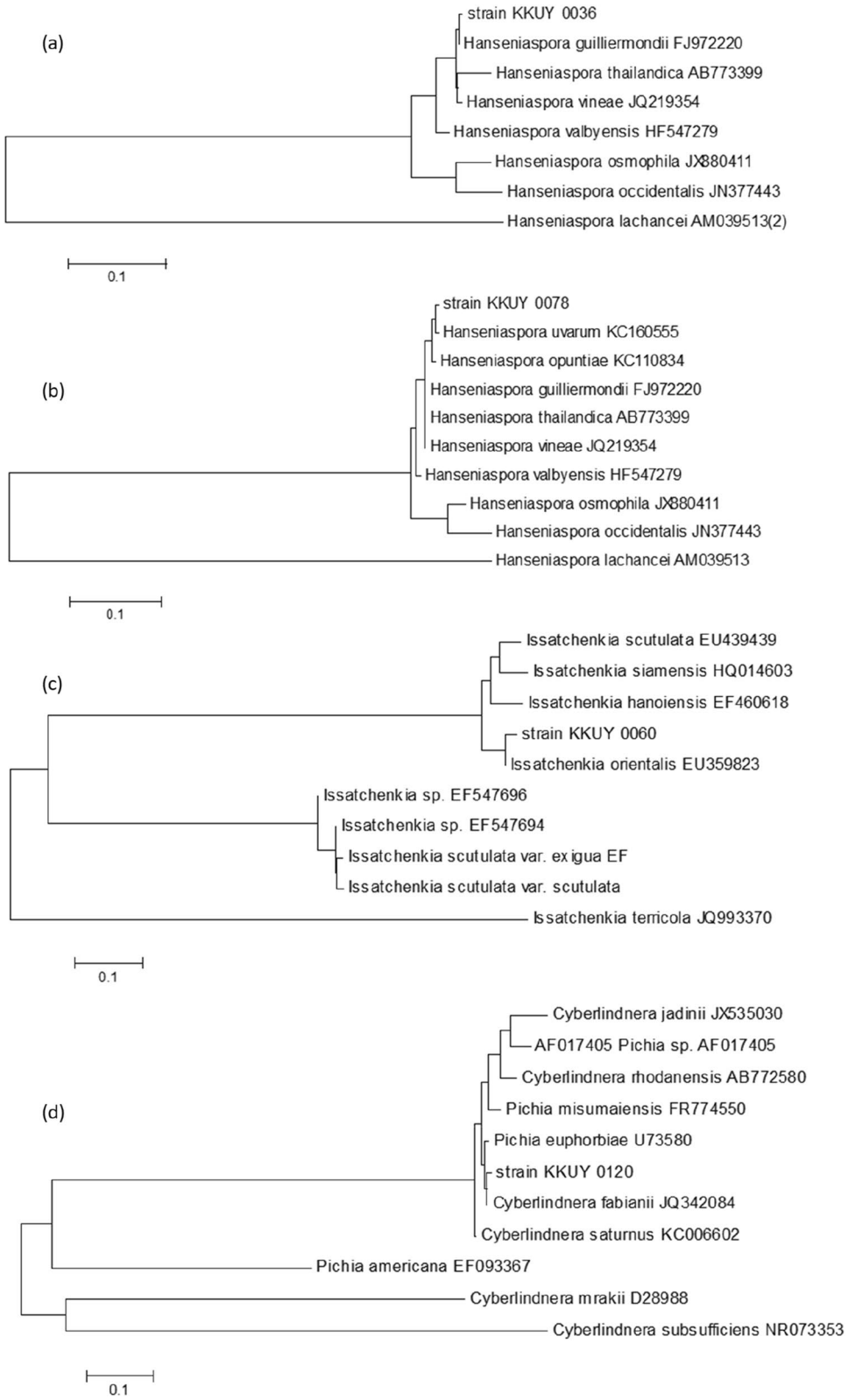

Fig. 2 Phylogenetic relationship between strains KKUY-0036, KKUY-0078, KKUY-0060, and KKUY-0120 and other 26S rDNA sequences of published strains. In the phylogenetic tree, the strains were clustered together with *Hanseniaspora guilliermondii* (**a**), *Hanseniaspora uvarum* (**b**), *Issatchenkia orientalis* (**c**), and *Cyberlindnera fabianii* (**d**), respectively, as one-clade segments corresponding to an evolutionary distance of 0.1 are shown with bars

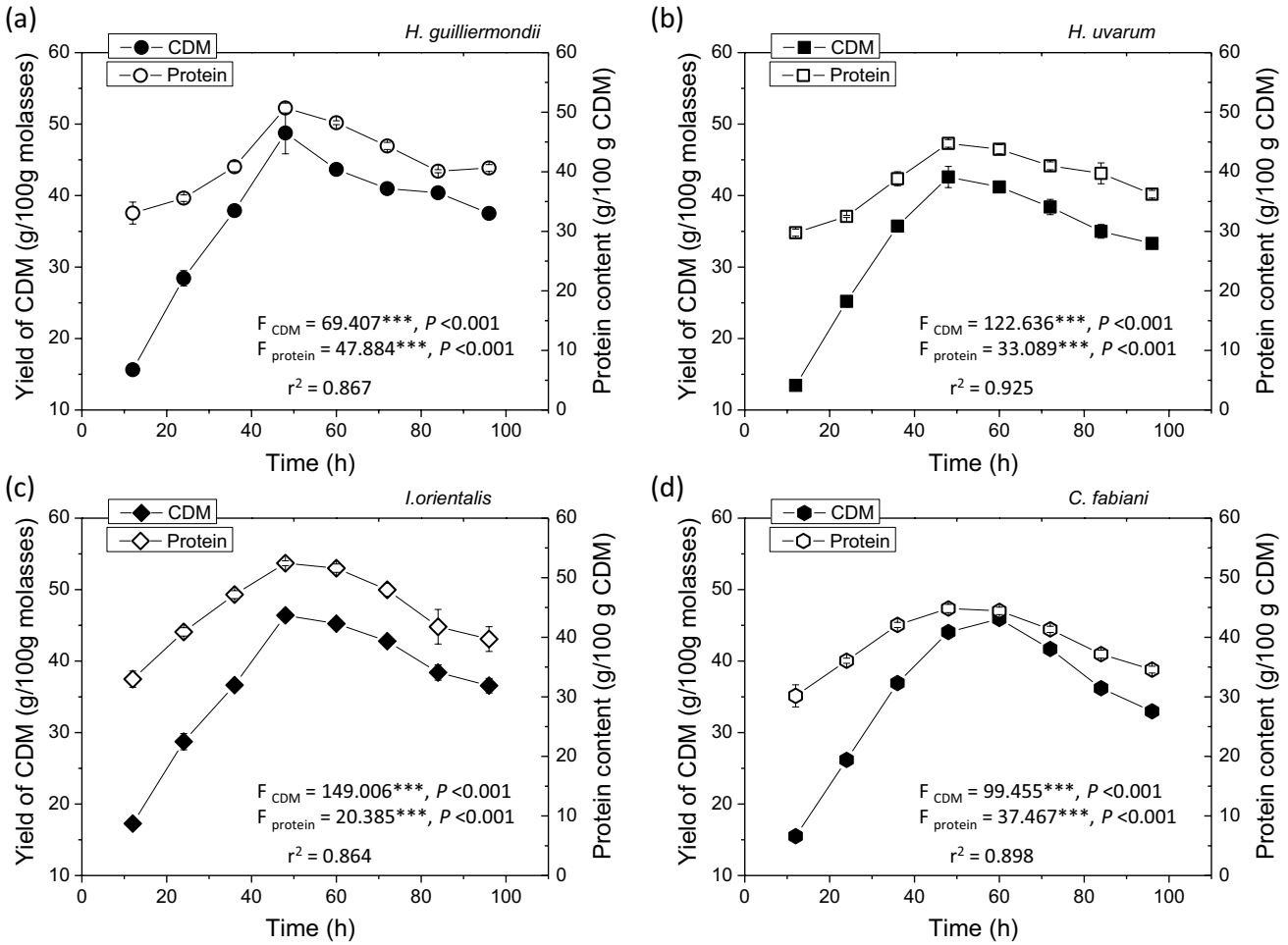

Fig. 3 Effect of incubation time on biomass production (g CDM/100 g WDM) and single-cell protein content (g protein/100 g CDM) during growth of *H. guilliermondii* (**a**), *H. uvarum* (**b**), *I. orientalis* (**c**), and *C. fabianii* (**d**), with date molasses as carbon source. Values are means of 3 replicates, and bars represent the standard error

Table 2 Effect of temperature on biomass yield (g CDM/100 g WDM) and single-cell protein (g protein/100 g CDM) by four yeast species grown on WDM. Values are means of 3 replicates ± standard error. Values in the same column followed by the same letter are not significantly different ($P < 0.001$)

Temperature	*Hanseniaspora guilliermondii*		*Hanseniaspora uvarum*		*Issatchenkia orientalis*		*Cyberlindnera fabianii*	
	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)
20 °C	24.50 ± 0.98^{a}	32.50 ± 0.90^{a}	23.87 ± 0.91^{b}	30.40 ± 0.59^{b}	39.00 ± 0.29^{b}	35.63 ± 0.43^{b}	20.40 ± 1.07^{b}	30.40 ± 0.59^{b}
25 °C	47.93 ± 0.72^{c}	$48.50 \pm 1.07b^{c}$	40.57 ± 0.75^{d}	43.40 ± 0.59^{c}	41.40 ± 0.12^{c}	45.07 ± 0.87^{c}	33.30 ± 0.96^{c}	43.40 ± 0.59^{c}
30 °C	48.17 ± 0.88^{c}	51.57 ± 0.86^{c}	46.17 ± 0.33^{e}	44.17 ± 0.88^{c}	51.00 ± 0.58^{d}	50.77 ± 1.10^{d}	37.50 ± 0.58^{d}	44.17 ± 0.88^{c}
35 °C	42.73 ± 0.39^{b}	46.50 ± 0.65^{b}	38.00 ± 0.06^{c}	43.23 ± 0.98^{c}	42.20 ± 0.12^{c}	45.53 ± 0.58^{c}	30.10 ± 1.10^{c}	45.20 ± 0.65^{c}
40 °C	27.53 ± 0.97^{a}	29.51 ± 1.56^{a}	4.87 ± 0.33^{a}	15.90 ± 2.27^{a}	35.60 ± 0.17^{a}	32.50 ± 0.0^{a}	13.63 ± 0.81^{a}	21.50 ± 0.58^{a}
F value	192.890***	64.047***	854.717***	100.014***	346.563***	115.148***	112.082***	249.509***
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
r^2	0.951		0.776		0.901		0.938	

CDM, cell dry mass; *WDM*, wasted date molasses

sufficient variation to define individuals at the species level [35, 45, 46].

3.3 Optimization of SCP production by the yeasts

The newly isolated yeast strains *H. guilliermondii*, *H. uvarum*, *I. orientalis*, and *C. fabianii* were further tested in terms of their SCP production capacity at various cultivation conditions, aiming to determining the optimal ones. In Fig. 3, the microbial growth and the variation of protein content for the four strains are illustrated versus time. It can be assumed that all four yeasts reached the maximum growth and protein content after 48–60 h of incubation. More specifically, the maximum growth of *H. guilliermondii* was achieved after 48 h as 48.76 g CDM/100 g WDM and the protein content was 50.70 %; however, *H. uvarum* showed 42.61 g CDM/100 g WDM of biomass and 44.76% of protein content at the same period. The biomass of *I. orientalis* was obtained as 46.42 g CDM/100 g WDM and its protein content was 52.43% after 48 h of incubation. *C. fabianii* produced its maximum productivity of biomass as 45.90 g CDM/100 g WDM after 60 h; however, the highest percentage of protein content (44.83%) was achieved after 48 h. There was a strong correlation between high amount of biomass and enrichment in protein content in the case of the four yeasts ($r^2 = 0.863–0.925$). The growing time is of great importance from the commercial point of view, wherein the faster the growing rate of the microorganism, the lower the cost of production of the SCP [46]. However, peak of growth varies greatly depending on many factors like yeast strain, substrate, temperature, pH, and inoculums size [23]; obtaining the peak of growth after 48 h in the case of our isolates makes them more appropriate than many other species, which reach their maximum growth after 60 h or more when they grow on biowastes. For example, Adoki [47] reported that the peak growth of *Candida* sp. grown on agro-wastes was obtained after 60 h. Dhanasekaran et al. [48] obtained the maximum growth of *Saccharomyces cerevisiae* and *Candida tropicalis* on pineapple waste after 7 days.

The production of SCP by the four selected yeast strains was further investigated at different incubation temperatures (20, 25, 30, 35, and 40 °C). The results clearly showed that the most appropriate temperature for SCP production was 30 °C; however, the degree of bioconversion of WDM towards biomass and SCP seemed to vary depending on the yeast species. The highest biomass yield was noted in the case of *I. orientalis* (51.00 g/100 g WDM) followed by *H. guilliermondii* (48.17 g/100 g WDM), whereas the highest protein content was detected in the case of *H. guilliermondii* (51.5%) (Table 2). The correlation between biomass and protein content was very strong ($r^2 = 0.901–0.976$). This confirms that the temperature affects biomass and protein content at the same level. Undoubtedly, temperature is one of the most important parameters influencing all activities of microorganisms [49], affecting the mechanisms and kinetics of transport of inorganic ions, amino acids, and sugars [50]. The effect of incubation temperature on SCP production has been studied for many different yeasts and fermentation media, indicating that the optimum temperature varies significantly among different strains. Murad et al. [15] suggested that 28 °C is the most favorable temperature for biomass production by *Kluyveromyces lactis* grown on whey permeate. Lee et al. [51] showed that the optimum temperature for thermotolerant *Candida tropicalis* used for SCP production was 38 °C. Rajoka et al. [52] studied the production of SCP by *Candida utilis* at different temperatures (20–45 °C) in a stirred fermentor and reported that the maximum production of crude protein was recorded when the fermentation temperature was maintained at 35 °C, whereas above 35 °C the production of crude protein decreased. Normally, high

Table 3 Effect of pH on biomass yield (g CDM/100 g WDM) and single-cell protein (g protein/100 g CDM) by four yeast species grown on WDM. Values are means of 3 replicates ± standard error. Values in the same column followed by the same letter are not significant ($P < 0.001$)

pH	*Hanseniaspora guilliermondii*		*Hanseniaspora uvarum*		*Issatchenkia orientalis*		*Cyberlindnera fabianii*	
	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)
4	53.00 ± 0.12^{d}	51.67 ± 1.12^{c}	48.07 ± 0.81^{c}	46.30 ± 1.11^{c}	54.20 ± 0.12^{c}	50.07 ± 1.11^{c}	43.73 ± 0.79^{d}	46.93 ± 0.81^{c}
5	48.20 ± 0.17^{c}	50.73 ± 1.12^{c}	45.73 ± 0.77^{c}	48.50 ± 0.58^{c}	49.40 ± 0.06	48.20 ± 1.20^{c}	40.73 ± 0.39^{c}	45.27 ± 0.39^{c}
6	42.60 ± 0.12^{b}	41.17 ± 0.88^{b}	41.40 ± 0.76^{b}	40.40 ± 0.59^{b}	$36.53 \pm 1.33b^{c}$	37.07 ± 1.27^{b}	35.07 ± 0.81^{b}	37.07 ± 1.27^{b}
7	25.73 ± 1.47^{a}	36.07 ± 0.43^{a}	30.40 ± 0.76^{a}	34.73 ± 1.18^{a}	20.17 ± 2.85^{a}	30.83 ± 0.20^{a}	19.83 ± 0.88^{a}	26.83 ± 0.88^{a}
F value	255.568***	66.112***	113.776***	46.471***	93.598***	77.566***	204.776***	105.565***
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
r^2	0.912		0.884		0.953		0.980	

CDM, cell dry mass; *WDM*, wasted date molasses

Table 4 Effect of substrate concentration on biomass yield (g CDM/100 g WDM) and single-cell protein (g protein/100 g CDM) by four yeast species grown on WDM. Values are means of 3 replicates ± standard error. Values in the same column followed by the same letter are not significant ($P < 0.001$)

WDM concentration (%)	*Hanseniaspora guilliermondii*		*Hanseniaspora uvarum*		*Issatchenkia orientalis*		*Cyberlindnera fabianii*	
	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)
10	45.80 ± 0.12^{ab}	45.40 ± 1.74^{a}	42.40 ± 0.67^{c}	$45.30 \pm 3.47b^{c}$	41.93 ± 0.78^{b}	48.17 ± 0.88^{bc}	35.83 ± 0.38^{a}	45.50 ± 1.73^{bc}
20	47.80 ± 0.17^{b}	46.97 ± 0.27^{a}	43.63 ± 0.81^{c}	$45.87 \pm 0.63b^{c}$	47.50 ± 0.58^{c}	$52.00 \pm 0.40b^{c}$	40.67 ± 0.29^{b}	49.70 ± 20.8^{d}
30	58.53 ± 3.64^{c}	51.80 ± 0.25^{b}	50.07 ± 0.57^{d}	48.53 ± 0.61^{d}	63.50 ± 1.18^{d}	48.40 ± 1.41^{d}	46.20 ± 0.30^{c}	46.17 ± 1.77^{bc}
40	43.20 ± 0.23^{ab}	44.30 ± 0.92^{a}	39.77 ± 0.59^{b}	40.53 ± 0.33^{ab}	$44.00 \pm 1.73b^{c}$	44.73 ± 1.18^{ab}	42.17 ± 1.17^{b}	41.30 ± 0.67^{ab}
50	40.40 ± 0.06^{a}	41.50 ± 1.00^{a}	35.40 ± 0.22^{a}	37.50 ± 0.53^{a}	37.20 ± 1.18^{a}	42.90 ± 1.23^{a}	35.47 ± 0.13^{a}	37.20 ± 1.18^{a}
F value	18.096	14.239	80.197	7.446	74.887	10.718	60.100	9.488
P value	< 0.001	< 0.001	< 0.001	< 0.01	< 0.001	< 0.01	< 0.001	< 0.01
r^2	0.849		0.833		0.419		0.363	

CDM, cell dry mass; *WDM*, wasted date molasses

Table 5 Effect of peptone concentration on biomass (g dry biomass/100 WDM) and single-cell protein (protein %) production by four yeast species from WDM. Values are means of 3 replicates ± standard error. Values in the same column followed by the same letter are not significant ($P < 0.001$)

Peptone concentration (g/L)	*Hanseniaspora guilliermondii*		*Hanseniaspora uvarum*		*Issatchenkia orientalis*		*Cyberlindnera fabianii*	
	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)
Control	45.80 ± 0.12^{a}	42.57 ± 0.35^{a}	41.20 ± 0.58^{a}	41.60 ± 0.71^{a}	43.27 ± 0.97^{a}	45.87 ± 0.32^{a}	36.07 ± 0.43^{a}	43.40 ± 0.90^{a}
1	49.42 ± 0.50^{b}	46.10 ± 0.26^{b}	44.23 ± 0.97^{b}	44.50 ± 1.00^{b}	68.16 ± 0.09^{b}	48.87 ± 0.32^{b}	42.07 ± 0.98^{b}	45.87 ± 0.32^{cd}
2	52.30 ± 0.00^{c}	53.17 ± 1.02^{c}	46.53 ± 0.55^{b}	50.33 ± 0.83^{c}	71.20 ± 0.05^{c}	52.77 ± 0.37^{c}	45.53 ± 0.58^{c}	45.20 ± 0.35^{d}
3	55.80 ± 0.36^{d}	53.23 ± 0.98^{c}	47.27 ± 0.62^{b}	51.53 ± 0.33^{c}	75.84 ± 0.14^{d}	54.30 ± 0.49^{cd}	47.50 ± 0.58^{c}	48.73 ± 1.14^{cd}
4	54.60 ± 0.70^{d}	51.87 ± 0.34^{c}	44.40 ± 0.49^{b}	50.30 ± 0.49^{c}	74.10 ± 0.90^{d}	54.60 ± 0.58^{d}	46.43 ± 0.46^{c}	44.45 ± 1.65^{cd}
5	52.67 ± 0.47^{c}	50.53 ± 0.34^{d}	44.03 ± 0.88^{b}	49.77 ± 0.27^{c}	74.57 ± 1.03^{d}	52.57 ± 0.32^{c}	45.20 ± 0.35^{c}	43.67 ± 0.84^{b}
F value	72.312	46.266	9.220	36.389	321.688	69.399	49.389	3.991
P value	< 0.001	< 0.001	< 0.01	< 0.001	< 0.001	< 0.001	< 0.001	< 0.05
r^2	0.907		0.716		0.881		0.400	

temperature can cause inactivation of enzymes of the metabolic pathway, while low temperature may not permit flow of nutrients across cell membranes, resulting in high demand for maintenance energy, and also at low temperature, the enzyme activities are expectedly low [53].

Weak acidity (pH 4–5) appeared as appropriate for the four yeasts to exert their maximum productivity of SCP. At pH 4, *I. orientalis* produced 54.20 g of biomass/100 g WDM and 50.07% of the protein content. *H. guilliermondii* produced 53.00 g/100 g WDM of biomass and 51.7% of protein content. The productivity of the other two yeasts was relatively low compared with either *I. orientalis* or *H. guilliermondii* (Table 3). The results of the current study are supported by the suggestion that acidic medium is more appropriate for the overall growth of yeasts [54, 55]. Pessoa et al. [56] reported that the maximum production of SCP by *Candida tropicalis* on diesel oil and sugarcane bagasse hydrolysate occurred at pH 6. Rajoka et al. [52] reported that pH 6 was the optimum for SCP production from rice by *Candida utilis*. Paraskevopoulou et al. (2003) found that the optimum pH for SCP production using the yeasts *Kluyveromyces*, *Candida*, *Saccharomyces*, and *Pichia* was 5.5. Interestingly, the weak acidity of the WDM (pH = 4.77) seems to make it an appropriate fermentation medium for efficient yeast growth, without any need for pH adjustment, contributing thus to the reduction of the production cost. Moreover, acidic pH of WDM could be further advantageous contributing to the control of possible condamination by bacteria by suppressing their growth [55].

Table 6 Effect of NH_4Cl concentration on biomass yield (g CDM/100 g WDM) and single-cell protein (g protein/100 g CDM) by four yeast species grown on WDM. Values are means of 3 replicates ± standard error. Values in the same column followed by the same letter are not significant ($P < 0.001$)

NH_4Cl concentration (g/L)	*Hanseniaspora guilliermondii*		*Hanseniaspora uvarum*		Issatchenkia orientalis		*Cyberlindnera fabianii*	
	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)	Biomass yield (g CDM/100 g WDM)	Protein content (g protein/100 g CDM)
Control	43.93 ± 0.98^{a}	41.30 ± 0.67^{a}	39.73 ± 0.23^{a}	40.83 ± 0.63^{a}	45.07 ± 1.45^{a}	42.77 ± 1.57^{a}	35.63 ± 0.43^{a}	41.30 ± 0.67
1	46.93 ± 0.81^{b}	44.40 ± 1.07^{a}	41.73 ± 0.96^{a}	42.07 ± 0.98^{a}	63.47 ± 0.93^{b}	46.87 ± 0.88^{b}	40.53 ± 0.33^{b}	42.07 ± 0.43
2	49.40 ± 0.49^{bc}	51.50 ± 0.66^{b}	44.40 ± 0.49^{b}	48.27 ± 0.79^{c}	66.97 ± 0.91^{bc}	50.67 ± 0.84^{c}	43.83 ± 0.67^{c}	43.40 ± 0.59
3	52.93 ± 0.63^{d}	51.97 ± 1.69^{b}	46.17 ± 0.33^{b}	48.40 ± 0.67^{c}	68.73 ± 0.79^{cd}	52.30 ± 0.67^{c}	45.42 ± 0.58^{cd}	46.73 ± 1.76
4	53.97 ± 0.39^{d}	49.27 ± 1.42^{b}	45.93 ± 0.72^{b}	46.40 ± 0.67^{bc}	71.97 ± 0.39^{d}	51.87 ± 0.33^{c}	46.73 ± 0.23^{d}	44.00 ± 1.41
5	51.57 ± 0.68^{cd}	49.40 ± 0.44^{b}	44.20 ± 0.35^{b}	44.83 ± 0.32^{b}	69.63 ± 0.57^{cd}	51.23 ± 0.61^{c}	43.97 ± 0.39^{c}	43.53 ± 1.67
F value	30.503	15.161	19.093	20.271	119.246	17.297	75.359	2.398
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	NS
r^2	0.796		0.820		0.870		0.550	

NS, the values are not significantly different

The substrate concentration to have a strong effect on the efficient growth of the yeast strains and the production of SCP. The highest concentrations observed for both biomass yield and protein content was in the range of 20 to 30%. Among the four yeasts, *I. orientalis* resulted to the highest biomass value (63.50 g/100 g WDM); however, the protein content was not so high (48.40%). Meanwhile, *H. guilliermondii* produced 58.53 g/100 g WDM and had the highest content of protein being 51.80% (Table 4). The results clearly showed that below or above the concentration range of 20–30% the productivity of biomass decreased by all yeasts. However, significant biomass yields are obtained even at higher concentrations indicating the osmo-tolerance characteristics of these strains. Indeed, extremely high sugar concentration is generaly reported to slowed down the growth rate, and eventually growth ceases due to plasmolysis of yeast cells [47].

The results showed that when the WDM medium was amended with peptone as a nitrogen source, the biomass and protein content were greatly enhanced by the four yeasts (Table 5). The highest biomass yield and protein content were detected in the case of *I. orientalis*. This microorganism produced 75.84 g of biomass/100 WDM containing 54.30% protein. *H. guilliermondii* occupied the second order regarding the production of biomass and protein content (55.80 g CDM/100 g WDM and 53% protein). NH_4Cl as a nitrogen source showed a lower stimulatory effect on the biomass and protein production compared with peptone. *I. orientalis* is still the highest producer of biomass and protein when it was grown on WDM amended with NH_4Cl; it produced 68.73 g of CDM/100 WDM and 52.30% protein (Table 6). *H. guilliermondii*, when supplemented with NH_4Cl as a nitrogen source, produced 52.93 g of CDM/100 WDM and 51.97% protein; however, the other two yeasts were involved in a relatively lower production of biomass and protein content compared with the first two strains. It is recorted that during the fermentation of substrates with low protein content, the supplementation with extra nitrogen is quite important for maintaining normal physiological processes and growth rates of the yeast [57, 58]. In accordance with our results, Akintomide and Antai [59] observed that crude protein yield increased significantly with the addition of extra nitrogen. It is indeed widely accepted that the availability of nitrogen is a major controlling factor of the final biomass yields, and consequently crude protein yields, since nitrogen is an essential element required for protein synthesis [60, 61]. Organic nitrogen such as peptone and yeast extract was reported to have better enhancement in protein synthesis by yeasts compared with by the inorganic sources [62].

3.4 Scale up experiments

Scaling up of the fermentations in a 7-L fermentor and at optimum growth conditions i.e. WDM 20%, pH 4, 30 °C, and 48 h, resulted to biomass growth and protein contents percentage to those obtained from the shaking flask experiments (Fig. 4). More specifically, the high-set yields were noted for *I. orientalis* and *H. guilliermondii* yielded to 75.82 g CDM/100 g WDM and 54.34% protein, and 55.82 g CDM/100 g WDM of biomass and 53.21% protein, respectively. Both *H. uvarum* and *C. fabianii* resulted to relatively lower biomass and protein content (47.53 g CDM/100 g WDM and 51.53–48.72% of protein). It is assumed that the similarity in SCP productivity in both shaking flasks and fermentor is because the yeast stains effectively consumed all available sugars in both cases and

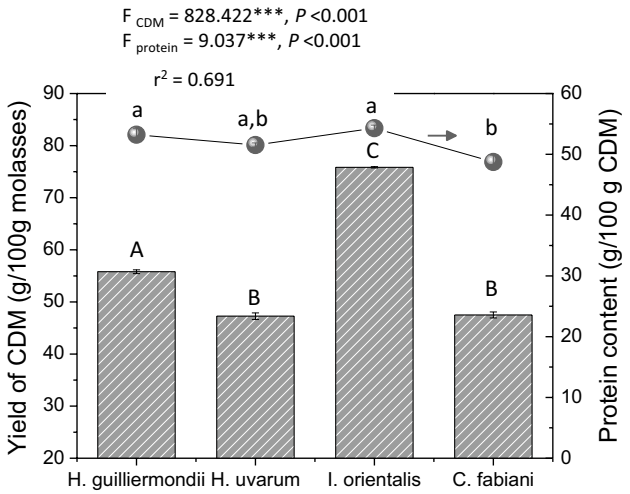

Fig. 4 Biomass yield (g CDM/100 g WDM) and single-cell protein content (g protein/100 g CDM) during growth of *H. guilliermondii*, *H. uvarum*, *I. orientalis*, and *C. fabianii* with date molasses (20%) as carbon source in a 7-L fermentor. Values are means of 3 replicates, and bars represent the standard error. Columns/symbols marked with the same letter are not significantly different ($P < 0.001$)

reached maximum growth and productivity based on the achieved yields and taking into account that protein percentage in yeasts generally ranges from 47 to 53% [55] it can be assumed the strains selected in the current study, are quite promising as protein producers and good candidates for commercial applications.

4 Conclusions

The study introduces four new effective yeasts and a new cost effective substrate (WDM) for the production of large amount of SCP. This substrate represents an excellent carbon source to support high rate microbial growth with efficient protein content that does not require any further pretreatment. However, amendment with an extra source of nitrogen seems to be essential for increased productivities. The consistency between the results obtained from shaking flasks and pilot experiment proved the efficiency of the yeasts in SCP production with a high protein content percentage (up to 54.3%). Via this study, these newly isolated yeasts are indicated as promising SCP producers for industrial and commercial production that could be used as supplement for animal feed. The study suggests further investigation to approve the benefit of these new yeasts as a safe protein-additive source in human dietary.

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Author contribution M.H. and S.A.: conceptualization; Y.S. and M.S.A.: methodology; M.H., S.A., and I.N.: data curation; M.H. and Y.S.: writing—original draft preparation; I.N. and G.L.: writing—review and editing; M.H. and G.L.: project administration.

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Data availability The data that support the findings of the current study are available from the corresponding author, M.H., upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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